Effects of Gamma Irradiation on Diphosphopyridine Nucleotide (Special Issue on Physical, Chemical and Biological Effects of Gamma Radiation, III)

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A chemical effect of small doses of γ-irradiation on reduced diphosphopyridine nucleotide in aqueous solutions was found significantly. The effect may be due to oxidation of the DPNH by an indirect action of γ-irradiation. Electron spin resonance spectra of irradiated DPN and of nicotine amide, a constituent of DPN, were investigated and formation of free radicals from irradiated coenzymes was proved. From the results in company with those of the previous paper, a reaction mechanism was proposed.

INTRODUCTION

In the course of studying chemical and biochemical effects of irradiation of γ-rays on enzymes, it became probable that not only the structure or the active center of enzyme molecules but also that of coenzymes should be caused destruction. Since coenzyme molecules are generally smaller in size and simpler in reaction modes than apoenzymes, reaction mechanisms would be relatively well established in the coenzymes. Effects of γ-irradiation on oxidized diphosphopyridine nucleotide (DPN) and reduced DPN (DPNH), therefore, were studied from a standpoint of radiation chemistry.

Chemical effects induced by gamma radiation on diphosphopyridine nucleotides in aqueous solutions may be divided into three types according as an irradiation dose :

1. A reaction induced by a relatively small dose of γ-rays (about 10^2 to 10^3 r) in aqueous solutions of reduced diphosphopyridine nucleotide (DPNH), is shown by the disappearance of absorption at 340 mμ, and is probably due to indirect oxidation of DPNH.

2. A reaction induced by a intermediate dose of γ-rays (about 10^3 to 10^4 r) occurs reductively only in an air-free aqueous solution of DPN under the existence of ethanol and other suitable organic compounds. The reduction is believed to proceed indirectly through a radical mechanism in the aqueous solutions.

3. Reactions induced by larger doses of γ-rays (about more than 10^4 r) in aqueous solutions of DPN, are direct and indirect radiolysis of DPN.

The reductive reaction of DPN by ethanol and other alcohols under the influence of X-irradiation was studied by Swallow[1] and Barron *et al.*[2], and the formation of a dimer of DPN through a radical mechanism was proposed by Swallow[3]. Similar results and some radiolytic reactions of DPN in aqueous solutions were reported by Tanaka *et al.*[4].

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In continuation of the previous investigation[4] further studies on the oxidative reaction of DPNH in aqueous solutions were carried out by smaller doses of γ-irradiation and formation of a free radical from a dried specimen of DPN as an intermediate is presumed to be possible to occur in this paper.

EXPERIMENTAL

Oxidized diphosphopyridine nucleotide, DPN, and reduced DPN, DPNH, (90% pure) were purchased from Sigma Chemical Co., U.S.A.. Nicotine amide was also a commertial specimen.

An ordinary procedure of deaeration was used for making dissolved air free from an aqueous solution. Relatively small doses of γ-rays (10^2 to 10^3 r) were irradiated by the Toshiba irradiation apparatus* in which 100 curie of cobalt-60 was equipped. For large doses of γ-irradiation (more than 10^3 r), the two-kilocurie cobalt-60 irradiation facility** was used with the technique employed previously[5]. Ultraviolet absorption spectra of the coenzymes were measured by a Beckman DU spectrophotometer. Electron spin resonance spectra were measured by a JES electron spin resonance spectrometer of Japan Electron Optics Laboratory Co., Ltd., Tokyo. The spectra were presented as a first derivative of the actual resonance curve. The observation was made on γ-irradiated, powdered DPN in vacuo at room temperature, and at a frequency of 9,000 Mc/sec.

RESULTS

(1) Radiolytic oxidation of DPNH induced by small doses of γ-irradiation in aqueous solutions.

When DPNH was exposured to γ-ray doses of 100 to 300 r both in its air-containing and air-free aqueous solutions, absorbancy of DPNH at 340 mμ decreased apparently. The experimental points are shown in Fig. 1. They fall more

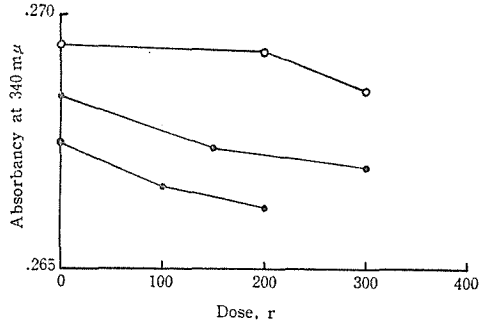

Fig. 1. The effect of very small doses of γ-rays on DPNH in air-containing (●) and air-free aqueous solutions (○).

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markable on the curve of air-containing aqueous solutions than of air-free solutions. Although DPNH was easily oxidizable spontaneously in air-containing aqueous solutions, a significant decrease was brought in these experiments.

The results obtained by the irradiation of γ-ray doses of 100 to 1500 r on similar solutions of DPNH were shown in Fig. 2, where linear decreases of the absorbancy at 340 mμ were found.

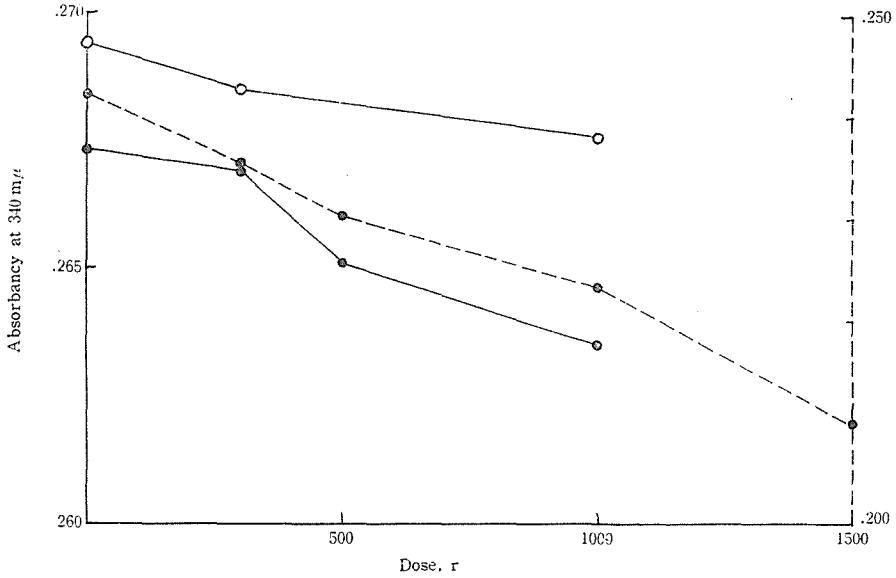


Fig. 2. The effect of small doses of γ-rays on DPNH in air-containing (●) and air-free (○) aqueous solutions.

(2) Formation of a free radical from DPN by γ-irradiation

An electron spin resonance spectrum of DPN irradiated with γ-ray dose of 1.2×10^4 r is shown in Fig. 3. The curve was obtained at a microwave frequency of 9,000 Mc/sec and at room temperature. Any resolvable hyperfine structure was not observed in its resonance. There is a broad component spacing of 165 gauss

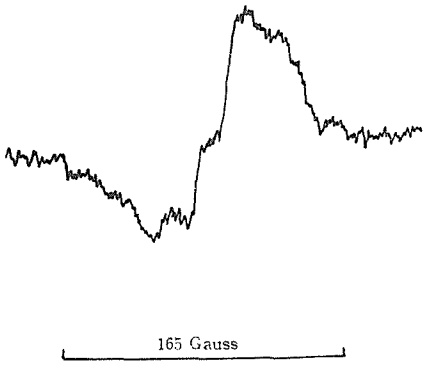


Fig. 3. Electron spin resonance of γ-irradiated DPN. Base marker is spaced 165 gauss apart. Dosage of γ-rays was 1.41×10^5 r.

Fig. 4. Electron spin resonance of γ-irradiated nicotine amide. Base marker is spaced 120 gauss apart. Dosage of γ-rays was 1.41×10^5 r.

and it may be resulted from an anisotropy in the g-factor.

The electron spin resonance of irradiated nicotine amide, is similar to that of DPN and have a broad component about 60 gauss in width, unresolved structure as shown in Fig. 4. This may be due to an anisotropy in the g-factor.

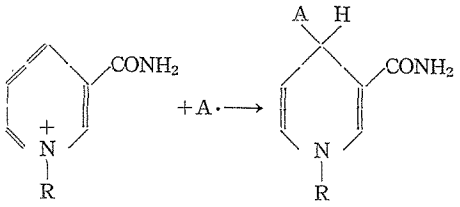
DISCUSSION

There are few previous remarks about chemical and biochemical effects of very small doses of irradiation on enzymes *in vitro*. The enzyme activity of aged myosin was reported to be inhibited when an aqueous solution of aged myosin was irradiated with one to ten roentogen doses of X-rays[6]. Yeast and liver alcohol dehydrogenases were also inactivated by irradiation of a few hundred roentogen dose of X- and γ-rays[7,8]. Here is shown an evidence that biological activity is lost when DPNH is irradiated even with small doses of γ-rays, in Figs. 1 and 2. This is able to be an biochemical parameter of a radiation effect *in vitro* as well as some biological parameters *in vivo*. These changes proceeded linearly to irradiation doses during the range of 10^2 to 10^3 r of γ-rays. These results seem to be due to an indirect action of radiation in aqueous solutions. It is doubtful that the DPN formed from DPNH by small doses of γ-irradiation in aqueous solutions has any activity as coenzyme.

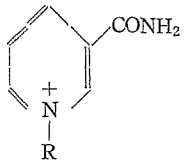
As mentioned above, one of the most interest reactions which are induced by 10^3 to 10^5 r doses of γ-irradiation may be the reduction of DPN in air-free aqueous solutions. This has already been found by Swallow[1], Barron[2], and Tanaka *et al.*[3]. Effective compounds, lactate and glutamate which are well known to be a substrate of the dehydrogenase, were reported to be available for the radiation reduction of DPN. The reduction product, however, do not have any coenzyme activities though it shows an absorption at 340 mμ. In this case, fluoresence which is shown by enzymatically reduced DPN could not been observed. It is doubtful that the product is DPNH. When at the 4-position of nicotine amide ring of DPN reduction was brought by an appropriate reducing agent, the specific absorption at 340 mμ may be observed. When at the 4-position of nicotine amide of DPN substitution of any other organic radical rather than H occurs, the similar absorption may be also observed at 340 mμ. In the present experiments, therefore, it would be expected to substitute organic radicals which are producible from alcohols, lactate, and other suitable compounds by irradiation at the 4-position of nicotine amide of DPN as follows:

$$AH+H\longrightarrow A\cdot+H_2 \qquad (1)$$

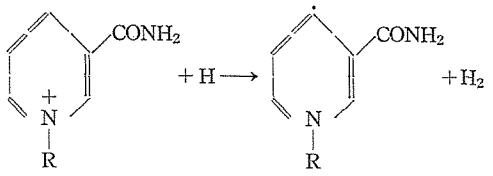
$$AH+OH\longrightarrow A\cdot+H_2O \qquad (2)$$



(3)

where A· presents an organic radical and  is DPN. Here was no

evidence that hydrogen atom is replacable to the organic radical[9,4]. Swallow proposed a recombination mechanism, (4)~(6), of DPN radicals as follows:



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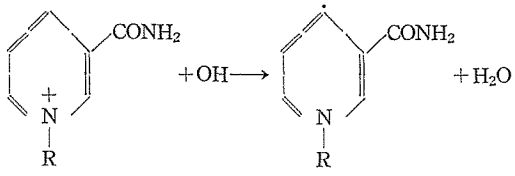
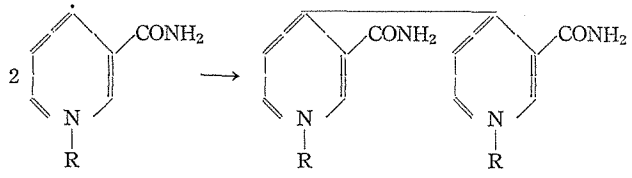
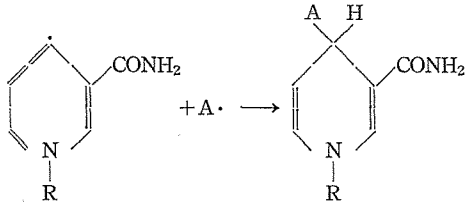


(5)



(6)



(7)

The evidence that the product is a dimer of DPN radicals, however, has not yet obtained. From the similarity of ESR spectra between irradiated DPN and nicotine amide, radical formation from DPN seems to be more plausible, through the radical mechanism, (4) (5) (7), and these reactions are appeared to proceed probably.

Radiation damages of DPN induced by large doses of γ-irradiation have been reported by the authors[4]. The very slight radiolytic damages of DPN were shown to occur from the decrease of absorption at 260 mμ and from inactivation of coenzyme activity. The radio-resistant nature of DPN is probably due to the conjugated structure of DPN, especially in the moieties of nicotine amide and adenine. However, specific ESR spectrum of adenosine, which was reported by Shields and Gordy[11], was not observed in the present observations. The nicotine amide ring may be more sensitive to radical formation than the adenine ring by irradiation. Many radiolytic products of DPN were separated from each other by an ion exchange column chromatography. Most of them have not yet been determined exactly except inorganic phosphate and a sugar component. Although radiolytic cleavage of a N-riboside bond was shown by a cyanide test any distinguishable sensitivities to radiation effects have not yet found in the coenzyme.

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